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• *BRIEF REPORTS* •

Effect of cytokine gene polymorphism on histological activity index, viral load and response to treatment in patients with chronic hepatitis C genotype 3

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Abstract

AIM: To investigate the association between cytokine gene polymorphism and disease status in chronic hepatitis C genotype 3 by liver biopsy, ALT, HCV RNA levels and response to treatment.

METHODS: Patients with chronic hepatitis C genotype 3 were analyzed for single nucleotide polymorphisms of interleukin (IL)-10, IL-1 beta, interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and transforming growth factor-beta (TGF-β) by polymerase chain reaction using sequence-specific oligonucleotide primers. Liver biopsies were assessed by modified histological activity index (HAI) scoring system using a scale of 0–18 for grading the necro-inflammatory activity and 0–6 for staging the fibrosis. HCV RNA levels were determined by bDNA assay. The patients were treated with interferon alpha and ribavirin for 6 mo. Sustained virological response was assessed 6 mo after the completion of the treatment.

RESULTS: Out of the 40 patients analyzed, 26 were males. Mean age was 40.5±12.5 years (range 18–65 years). The frequencies of different dimorphic polymorphisms based on single nucleotide substitution were as follows: IL-10-1082 G/A 85%, A/A 12.5%, G/G 2.5%; IL-10-819 A/C 87.5%, C/C 10%, A/A 2.5%; IL-10-592 C/A 72.5%, C/C 27.5%; IL-1 C 90%, U 10%; IFN-874 T/A 50%, T/T 27.5%, A/A 22.5%; TNF-308 A/G 95%, G/G 5%; TGF-10 T/C 52.5%, C/C 35%, T/T 12.5%. The mean grades of necro-inflammatory activity of different genotypes of IL-10 at promoter site -1082 were A/A = 3.6, A/G = 5.0, and G/G = 10.0 and the difference was significant (P = 0.029). The difference in the stage of disease at a scale of 0–6 was A/A 0.8, A/G 2.3, and G/G 4.0 (P = 0.079). The difference in the HAI seemed to be related to the presence of allele -1082G. For IL-10 -819 genotypes, mean scores of fibrosis were A/A = 6.0, A/C = 2.2, and C/C = 1.0 (P = 0.020) though the inflammatory activity was not much different. No significant differences in HAI were noted among polymorphisms of other cytokines. Moreover, ALT and HCV RNA levels were not significantly different among different cytokine polymorphisms. There was a significant correlation of HAI and HCV RNA levels with the duration of disease. TGFβ -10 genotype CC patients had a better end of treatment response than those with other genotypes (P = 0.020). Sustained virological response to the treatment was not influenced by the cytokine polymorphism. No effect of other factors like viral load, degree of fibrosis, gender, steatosis, was observed on sustained virological response in this population infected with genotype 3.

CONCLUSION: There is no significant correlation between cytokine polymorphisms and HAI except for the polymorphisms of anti-inflammatory cytokine IL-10, which may influence hepatic inflammatory activity and fibrosis in patients with chronic hepatitis C genotype 3. Sustained virological response in this genotype does not seem to be influenced by cytokine gene polymorphisms.

Key words: Interleukin; Interferon gamma; Tumor necrosis factor alpha; Transforming growth factor; Cytokines; Gene polymorphism; Hepatitis C; Alanine aminotransferase; Liver biopsy

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INTRODUCTION

HCV infection is a leading cause of chronic liver disease worldwide. The infection leads to viral persistence and chronic disease in a very high proportion of cases. Pathogenesis of liver injury is not fully understood. There is a complex relationship between HCV and its host. Liver lesions could be the result of immune

responses or cytopathic action of the virus. Cytotoxic T cells and cytokines produced by both CD4+ (T helper) and cytotoxic T cells may be responsible for much of the damage that occurs in the livers of infected patients[1].

Two distinct patterns of cytokine production may occur[2]. Type 1 responses are characterized by production of interleukin-2 (IL-2), tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ), which are prime and maintain antigen-specific cellular immunity[3,4] and are important in defense against viruses. Type 2 responses are characterized by IL-4, IL-5, and IL-10, which promote humoral immune responses. An imbalance in helper T-cell type 1 (Th1) and type 2 (Th2) cytokines is suggested to play an important role in the pathogenesis of chronic hepatitis C. The progressive liver injury seen in chronic HCV infection is associated with the upregulation of intrahepatic Th1-like cytokines. Intrahepatic IFN-γ and IL-2 mRNA expression is upregulated in chronic hepatitis C, while the expression of IL-10, a Th2-like cytokine, is downregulated[5] .

Intrahepatic CD4+ T cells play a pathogenetic role in the hepatic injury of HCV infection[6]. Vigorous HCV-specific CD4+ Th1 response, particularly against the nonstructural proteins of the virus, may be associated with viral clearance and protection from disease progression[7]. Patients without viremia after HCV infection frequently have strong Th lymphocyte responses of the Th1 type to multiple HCV antigens many years after the onset of infection, whereas antibody responses are less marked. These results suggest that control of HCV replication may depend on effective Th lymphocyte activation[8,9]. There is also an enhanced Th2 response during chronic HCV infection, which may partly be responsible for the persistence of HCV infection.

In addition to the altered intrahepatic cytokine expression, there might be a significant correlation between circulating cytokines and degree of inflammation in the liver. One study has shown such a correlation between baseline TNF levels and histologic grading score of hepatitis[10]. The maximal capacity of cytokine production varies between individuals and may correlate with polymorphism in cytokine gene promoters. The objectives of our study were to analyze the role of allelic or genotype variations of IL-10, IL-1 beta, IFN-γ, TNF-α and TGF-β and its association with hepatocellular injury as suggested by liver biopsy and ALT and treatment outcome. We selected genotype 3 for this study which is the main genotype in our country.

MATERIALS AND METHODS

Out of the 40 patients analyzed, 26 were males. Mean age was 40.5±12.5 years (range 18-65 years). The participants did not receive interferon therapy and had neither co-infection with human immunodeficiency virus and hepatitis B virus nor other associated forms of chronic liver disease.

Quantitative serum HCV RNA was determined by bDNA assay (Bayer, USA) according to the manufacturer' s instructions. The minimum quantification limit of the assay was 3 000 HCV RNA copies/mL serum. HCV genotyping was performed using PCR and reverse hybridization assay (Innogenetics, Belgium). All patients included in this study were of HCV genotype 3. For cytokine gene polymorphism, DNA was extracted by proteinase K digestion from peripheral mononuclear cells, followed by phenol chloroform extraction and ethanol precipitation. After amplification polymorphisms in IL-10 (592, 819, 1082), IL-1β (-511), IFNγ (874), TNFα (308) and TGF (codon25) were examined as described previously[11-13]. The number in parenthesis indicates the location of the polymorphism on the DNA sequence. Briefly, single nucleotide polymorphisms (SNPs) were determined using sequence specific oligonucleotide primers. Each PCR reaction consisted of 1× PCR buffer, 0.2 mmol dNTPs. Concentration of $MgCl_2$ varied with the type of SNP examined, 50 ng of each polymorphism specific primer, 1 U of *Taq* polymerase in a final volume of 10 μL. To monitor PCR inhibition, growth hormone gene was simultaneously amplified as an internal control. Thermal cycling was performed in a Perkin-Elmer 9700 thermal cycler. Following PCR amplification, amplicons were stained with ethidium bromide and visualized on a UV transilluminator. The size of product generated in each PCR assay was ascertained and scored as positive/negative for the presence/absence of a particular polymorphism. The PCR product obtained with IL-1β-511 specific primers was digested with the restriction enzyme *Ava*II. The digested product was then visualized for the presence of restriction fragments.

Liver biopsy specimens were analyzed by a single pathologist, who was unaware of the patient's identity, treatment regimen, response, or timing of the biopsy relative to the treatment. Liver biopsies were assessed by modified histological activity index (HAI) scoring system[14] using a scale of 0-18 for grading and 0-6 for staging. Degree of steatosis was scored at a scale of 0-3. Presence or absence of lymph follicles was also documented.

Duration of disease was determined by calculating time interval from the exposure to a possible risk factor. Patients were treated with standard doses of interferon alpha (3 mega units subcutaneous, thrice a week) and ribavirin (800-1 200 mg/d) for 6 mo and followed up for another 6 mo. HCV RNA was repeated 6 mo after the treatment to document sustained response.

Statistical analysis

Statistical analysis was performed by two-tailed tests. *P* values were calculated by one-way analysis of variance (ANOVA), Pearson's χ^2 and Spearman's rho correlation tests. $P<0.05$ was considered statistically significant.

RESULTS

The frequencies of different dimorphic polymorphisms based on single nucleotide substitution were as follows:

IL10-1082 G/A 85%, A/A 12.5%, G/G 2.5%; IL10-819 A/C 87.5%, C/C 10%, A/A 2.5%; IL10-592 C/A 72.5%, C/C 27.5%; IL-1 C 90%, U 10%; IFN-874 T/A 50%, T/T 27.5%, A/A 22.5%; TNF-308 A/G 95%, G/G 5%; TGF-10 T/C 52.5%, C/C 35%, T/T 12.5%. The mean grades of necro-inflammatory activity at a scale of 0-18 for different genotypes of IL10 at promoter site -1082 were A/A = 3.6, A/G = 5.0, and G/G = 10.0 and the difference was significant (P = 0.029). The difference in the stage of disease at a scale of 0-6 was A/A 0.8, A/G 2.3, and G/G 4.0 (P = 0.079). This difference in the HAI seemed to be related to the presence of allele -1082G. For IL 10 -819 genotypes the mean scores of fibrosis were A/A = 6.0, A/C = 2.2, and C/C = 1.0 (P = 0.020) though the inflammatory activity was not much different.

No significant differences in the degree of necro-inflammatory activity and fibrosis were noted among the polymorphisms of other cytokines (Table 1). Moreover, ALT and HCV RNA levels were not significantly different among different cytokine polymorphisms. There was a significant correlation of duration of disease with grade and stage of disease and HCV RNA levels (P = 0.017, 0.018, and 0.015 respectively with Spearman's rho test).

Out of the 40 patients, 34 remained under the follow-up. These patients completed the 6-mo treatment with interferon and ribavirin. HCV RNA was repeated 6 mo after the treatment to document the sustained response and the effect of cytokine gene polymorphism. TGFβ -10 genotype CC patients had a better end of treatment response than those with other genotypes (P = 0.020), though there was no difference in the sustained virological response. No effect of other factors like viral load, degree of fibrosis, gender, steatosis, was observed on the sustained virological response in this population infected with genotype 3.

DISCUSSION

Approximately 80-90% of patients acutely infected with hepatitis C virus develop persistent infection, about one-half of them have elevated transaminases indicative of ongoing liver inflammation[15]. In the context of an inflammatory response against the virus, variable cytokine response of the host may be responsible for the variable liver damage. Moreover, the cause of viral persistence during HCV infection may be the development of a weak antiviral immune response to the viral antigens, with corresponding inability to eradicate infected cells or insensitivity of the virus to such cytokines or insufficient production of cytokines[16]. Thus, the continuing inflammation results in liver damage in the absence of complete virologic recovery[17].

Studies have shown that active liver injury in chronic hepatitis C patients is associated with increased circulating Th1 cytokine IL-2 but not with Th2 cytokine IL-10[18]. It has also been shown by some workers that serum alanine transaminase and the hepatic fibrosis levels are related directly to the frequencies of peripheral memory effector CD8(+) T cells producing IFN-γ (Tc1), but inversely to the frequencies of those producing both IL-4 and IL-10 (Tc2)[19]. Moreover, most liver-infiltrating T cells in chronic hepatitis C are type 1 cells. Studies to date in liver tissue showed that intrahepatic mRNA for type 1-like cytokines, such as IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ were upregulated in chronic HCV infection[20]. The level of expression of type 1 cytokines, such as IL-2 and IFN-γ, is correlated with the degree of histologic injury as well as the likelihood of non-responsiveness to IFN-α therapy[21]. The presence of an ongoing cellular immune response probably also contributes to the process of hepatic fibrosis. Kupffer cells can be activated by the production of cytokines such as TNF-α, which in turn produce TGF-β[22].

Serum levels of different cytokines may not give the true picture of what is going on in the liver. For example, the mean IL2Rs and IFN serum levels are much higher in patients with anti-HCV than in the control group,

Table 1 Statistical significance of effects of cytokine gene polymorphisms on different parameters

Cytokine gene polymorphism	ALT (upper limit normal)	HCV RNA level	Grade of inflammation (0-18)	Stage of fibrosis (0-6)	Steatosis (0-3)	Lymph follicles	End of treatment response	Sustained response
IL 10 -1082 (GA, AA, GG)	0.636	0.241	0.029	0.079	0.267	0.848	0.078	0.331
IL 10 -819 (AC, CC, AA)	0.794	0.781	0.412	0.020	0.57	0.295	0.063	0.331
IL 10 -592 (CA, CC)	0.582	0.198	0.243	0.281	0.551	0.626	0.416	0.283
IFNγ -874 (AA, TT, TA)	0.389	0.848	0.919	0.921	0.955	0.382	0.21	0.933
TNFα -308 (AG, GG)	0.952	0.436	0.777	0.307	0.32	0.85	0.674	0.451
TGFβ -10 (CC, TC, TT)	0.734	0.72	0.386	0.959	0.232	0.643	0.026	0.206
IL 1 (C/U)	0.144	0.591	0.964	0.826	0.326	0.836	1.00	1.00

P values were calculated by ANOVA and Pearson' s χ^2 test. Statistically significant values. IL: interleukin; IFNγ: interferon gamma; TNFα: tumor necrosis factor alpha; TGFβ: transforming growth factor beta.

whereas the mean IL4 and IL6 levels are lower in patients infected with HCV[23]. Another study shows the higher levels of serum IL-1β, IL-4 and IL-6 (0.221, 0.104 and 1.393 pg/mL) in all HCV patients than in healthy adults (0.188, 0.025 and 0.600 pg/mL)[24].

Sustained response to interferon and ribavirin, defined as undetectable HCV RNA at 6 mo after discontinuation of therapy, is achievable in 30-60% of treated patients[25,26]. Predictors of response include viral factors such as viral genotypes, viral load and early disappearance of HCV RNA after initiation of therapy, while the host factors include gender, age and degree of fibrosis[25-27]. In recent years, increasing attention has been drawn to the role of host variation in cytokine levels in inflammatory and immune responses. Polymorphisms in genes encoding immunoregulatory proteins, proinflammatory cytokines, and fibrogenic factors may affect the production of these factors and influence disease progression in patients with chronic liver disease due to alcohol, primary biliary cirrhosis, or hepatitis C[28].

There might be an association of cytokine gene polymorphism and susceptibility to hepatitis C infection. Hohler *et al*[29] have reported such an association with the polymorphism at the TNFα promoter. The TNF promoter variants TNF2 (-238A) and TNF3 (-308A) confer a 3.2-fold and 5.1-fold risk of cirrhosis respectively (P = 0.03 for both). Reciprocal effects have been observed with several TNF alleles and haplotypes defined by the -238 G/A and -308 G/A dimorphic sequences, thus polymorphisms in the TNF alpha promoter appear to be associated with the variability in the histological severity of chronic hepatitis C infection[30]. In our study done on hepatitis C genotype 3 patients, no such effect of TNFα -308 variability was observed.

Interleukin (IL)-10 is a cytokine that downregulates the proinflammatory response and has a modulatory effect on hepatic fibrogenesis and is a potent anti-inflammatory Th2 cytokine that downregulates the expression of major histocompatibility complex (MHC) class I and class II molecules, as well as the production of Th1 cytokines[31-36]. IL-10 levels differ widely between individuals, possibly because of polymorphisms in the promoter region of the IL-10 gene[37,38]. Specifically, three SNPs in the promoter (at positions -1082, -819, and -592 relative to the transcription start site) form three SNP combinations (ATA, ACC, GCC), which are associated with differential IL-10 expression[38-40]. It is reasonable to assume that hepatitis C patients who produce high levels of IL-10 have less hepatocellular injury and less ability to control infection and patients with low secretion of IL-10 have a better ability to eliminate the hepatitis infection. Perhaps low IL-10 production can skew the immune system into the Th1 type of response, facilitating the clearance of viral load. -1082A allele is associated with reduced IL-10 production *in vitro*[39]. In our study, individuals who were homozygous for IL-10 AA at position -1082 had a lower HAI.

It has been shown that hepatitis C patients, genotyped as high IL-10 producers, have a poor response to IFN-α therapy[40]. Such polymorphisms may also predict the sustained viral response to antiviral therapy[41]. These patients may benefit from additional treatment strategies designed to enhance T-helper type 1 (Th1) response. In one study, the interleukin-10 -1082 G/G genotype was identified more frequently in patients than in controls (P = 0.048). The patients exhibiting transforming growth factor-beta 1+29 (codon 10) C/C genotype variables were less likely to respond to treatment than patients with the T/T or T/C genotypes[42]. Liver transplant recipients, who are genotyped as having a low production profile of IL-10, are more prone to rejection and less likely to have hepatitis C recurrence[43]. The IL-1beta-31 genotype T/T or the IL-1beta-511/-31 haplotype C/T is associated with the presence of HCC in Japanese patients with chronic HCV infection[44].

However, not all the studies favor such effect of polymorphisms. Three members of the interleukin-1 gene family (IL-1A, IL-1B and IL-1RN), three polymorphic sites in the interleukin-10 gene promoter (-1082, -819, -592) and two in the TNF-α promoter (-308, -238) were studied in two independent DNA banks, each with appropriate controls. Standard PCR-based genotyping techniques were used. No significant difference in the distribution of any of the polymorphisms has been found in either study set[45].

We, in this study, determined SNP at position -1082, -819 and -592 in case of IL-10, -874 for IFN-γ, -308 for TNF-α, -10 for TGF-β and IL-1 C/ U and analyzed the frequency of their distribution and correlation with the ALT and HCV RNA levels, HAI and response to treatment. In our series, we selected genotype 3 patients because this is the main genotype in our region and not enough data are available on the influence of host cytokine gene polymorphisms of this viral genotype. Another reason of selecting a single genotype was to make the group uniform as different genotypes have different response rates to antiviral therapy and influence of cytokines may also be different. We could not find any significant difference in the cytokine genotype profile while analyzing different variables except for some influence of polymorphisms of IL-10 on liver histology. These polymorphisms did not modulate the response to interferon plus ribavirin therapy. This may be due to the small sample size or the fact that viral genotype 3 is easy to treat genotype in any case.

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